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RESEARCH PAPERS

Protection of Growth and Photosynthesis of *Brassica juncea* Genotype with Dual Type Sulfur Transport System against Sulfur Deprivation by Coordinate Changes in the Activities of Sulfur Metabolism Enzymes and Cysteine and Glutathione Production[1]

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Abstract—Mustard (*Brassica juncea* L. Czern and Coss.) cvs. Pusa Jai Kisan (with low-affinity S transporter (LAT) system) and Pusa Bold (with dual, low- and high-affinity transporters (LAT + HAT) system) were supplied with 0 or 1 mM S in hydroponics culture, and the coordinate changes in growth traits (plant dry weight and leaf area), photosynthetic traits (photosynthetic rate, intercellular CO_2, F_v/F_m, and chlorophyll content), activities of key enzymes of sulfur metabolism, such as ATP-sulfurylase (ATP-S), serine acetyltransferase (SAT), and glutathione reductase (GR), and the contents of cysteine (Cys) and glutathione (GSH) were studied in 30 days after sowing. The results showed that cv. Pusa Jai Kisan was more sensitive to S deprivation than cv. Pusa Bold. In cv. Pusa Jai Kisan, S deprivation resulted in a stronger decrease of plant growth and photosynthetic traits, Cys and GSH contents, and a notable decline in activity of ATP-S. S deprivation up-regulated GR activity to a greater extent in cv. Pusa Bold. In contrast, despite the activity of SAT, an enzyme involved in the final step of Cys biosynthesis, was increased in cv. Pusa Jai Kisan stronger than in cv. Pusa Bold under S-deprivation, it could not be translated into the increase in Cys and, thus, GSH contents and a consequent improvement in growth and photosynthesis. The study demonstrated that cv. Pusa Bold (with LAT + HAT) can be a promising cultivar for activation of Cys and/or GSH biosyntheses and increased plant tolerance to S-deprivation conditions.

Keywords: Brassica juncea, ATP-sulfurylase, serine acetyltransferase, glutathione reductase, cysteine, glutathione, photosynthesis, sulfur deprivation, sulfur transporters.

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INTRODUCTION

Sulfur (S) is an essential nutrient required for plant growth and development [1, 2]. Sulfur deficiency in soil is increasing globally due either to the use of high-analysis low-S containing fertilizers, the decreasing use of S-containing fungicides and pesticides, high yielding varieties, intensive agriculture, and/or the reduction of SO_2 emission from industrial sources [3]. Sulfur is imported from soil into the plant in the form of inorganic sulfate. The assimilation of sulfate in plants is initiated by its activation, and the reaction is catalyzed by the enzyme ATP-sulfurylase (ATP-S) and finally leads to the formation of cysteine (Cys) [1, 3]. Cys serves as a precursor for a range of further reduced S-containing compounds, including glutathione (GSH). GSH represents a major S storage capacity in plants and performs several essential functions within the plant cell, serving as a redox regulator and antioxidant [4–6]. As a component of the ascorbate–GSH cycle, it takes part in the removal of H_2O_2 excess, in a reaction, in which GSH is oxidized to glutathione disulfide (GSSG). The high ratio of GSH to its oxidized form, GSSG, occurring under optimal growth conditions can be restored by means of higher glutathione reductase (GR) activity, increased GSH synthesis, decreased GSH degradation, or the transport of GSH and GSSG [7]. In addition, by affecting

[1] This text was submitted by the authors in English.

Abbreviations: ATP-S—ATP-sulfurylase; Chl—chlorophyll; DTNB—dithiobis-2-nitrobenzoic acid; GSH—glutathione (reduced); HAT—high-affinity transporter; LAT—low-affinity transporter; OAS—*o*-acetylserine; SAT—serine-acetyltransferase.

general plant growth and development, S deprivation has been shown to affect oxidative stress protective pathways in plants [8]. Earlier, we have reported that increases in Cys and GSH pools help plants to cope with cadmium-induced oxidative stress in a mustard genotype with high photosynthetic potential and ATP-S activity [9]. In addition, we have also reported that GSH is positively correlated with photosynthesis and plant dry weight in *Brassica campestris* and a proper S supply to plants may expand the GSH pool [2]. Thus, acclimation to S deprivation requires responses that allow essential reactions of primary metabolism to continue and enable plant to tolerate S deficit [10]. However, plants apparently have developed a regulatory mechanism that essentially activates the uptake and subsequent assimilation of sulfate to yield oxidative stress protectants permitting for survival under S-deprivation conditions [8]. Because ATP-S, a rate-limiting enzyme in S assimilation, regulates Cys (and thus GSH) biosynthesis and serine acetyltransferase (SAT) is an enzyme involved in the final step of Cys biosynthesis, it was hypothesized that mustard genotype with the efficient S-uptake system would modulate ATP-S, SAT, and GR enzymes for the production of Cys and GSH and, thus, to maintain high photosynthesis and growth under S deprivation.

Although there are several reports on the responses of crop plants to S availability in terms of changes in various aspects of plant growth and development, there appears to be a shortage of literature with regard to the coordinate changes in key enzymes of sulfur metabolism, such as ATP-S, SAT, and GR involved in the production of Cys and GSH, and their impact on the protection of growth and photosynthesis in mustard genotypes that differ in S transporter systems under S deprivation.

In this work, we have expanded our knowledge of adaptive changes in important oilseed crop mustard with different types of S-transporter system [11]. We addressed changes in energy production (photosynthesis) and oxidative stress protection (antioxidants).

MATERIALS AND METHODS

Plant growth conditions and treatments. Seeds of mustard (*Brassica juncea* (L.) Czern. and Coss) genotypes were sown in pots with moist vermiculite and germinated at a constant temperature of 20°C, relative humidity of 75%, and a 16-h light period (280–300 μmol/(m^2 s). After 6 days, plants were grown hydroponically for 30 days in nutrient solution containing 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_3PO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnCl_2$, 2 μM $ZnCl_2$, 0.5 μM $CuCl_2$, 0.5 μM $(NH_4)_6Mo_7O_{24}$, 20 μM $Na_2FeEDTA$. The pH of the solution was adjusted to 5.5 with KOH. Sulfate was supplied as $MgSO_4$ at two levels, viz., 0 mM (S-deprived) and 1 mM SO_4^{2-} (S-sufficient). Mg^{2+} was maintained at 1 mM by the addition of $MgCl_2$ in the two treatments. The nutrient solution was replaced weekly. The pots were arranged in a randomized block design, and three replicates were used. All the measurements were performed in 30 days after sowing.

Determination of growth and photosynthetic traits. Leaf area was measured with a LA 211 leaf area meter (Systronics, India), while plant dry weight was estimated after drying the sample at 80°C in an oven for 48 h. The net photosynthetic rate (P_N) and intercellular CO_2 concentration (C_i) were measured on fully expanded leaf (third from the top) using a Li-COR 6200 infra red gas analyzer (Li-COR, United States) at 11:00–12:00 a.m. at light saturating intensity. Chlorophyll (Chl) content was estimated according to Hiscox and Israelstam [12] using dimethyl sulfoxide (DMSO) while, the Chl fluorescence index (F_v/F_m) was measured in vivo after 0.5 h of dark adaptation using an OS-30p Chl fluorometer (Opti-Sciences, United States).

Assays of ATP-sulfurylase, serine acetyltransferase, and glutathione reductase activities. ATP sulfurylase activity was measured using molybdate-dependent formation of pyrophosphate as described by Khan et al. [9]. Fresh leaf tissues (0.5 g) were rapidly ground at 4°C in a buffer consisting of 10 mM Na_2EDTA, Tris–HCl (20 mM, pH 8.0), 2 mM DTT, and approximately 0.01 g/ml of insoluble polyvinyl pyrrolidone, using 1 : 4 (w/v) tissues to buffer ratio. The homogenate was strained to gauze and centrifuged at 20000 *g* for 10 min at 4°C. The supernatant (crude extract) was used for the ATP-S assays. The reaction was initiated by adding 0.1 ml of crude extract to 0.5 ml of the reaction mixture, which contained 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2ATP, and 0.032 units/ml of sulfate-free inorganic pyrophosphatase in Tris–HCl buffer (80 mM, pH 8.0). Another aliquot from the same extract was added to the same reaction mixture without Na_2MoO_4. Incubations were carried out at 37°C for 15 min, after which phosphate was determined spectrophotometrically (SL164, Elico, India). The ATP-S-dependent formation of pyrophosphate was estimated from the difference between the two figures.

Serine acetyltransferase activity in the crude leaf extract was determined by the method of Kredich and Tomkins [13]. Fresh leaf tissues (0.5 g) were ground with a chilled mortar and pestle in 2 ml of ice-cold extraction buffer (Tris–HCl (100 mM, pH 8.0), 100 mM KCl, 20 mM $MgCl_2$, 1% Tween 80, and 10 mM DTT). The samples were transferred to microcentrifuge tubes and spun at 11600 *g* for 10 min at 4°C. The supernatant obtained was used for SAT assay. The enzyme reaction mixture 1 ml in volume contained 0.1 mM acetyl-CoA, 50 mM Tris–HCl (pH 7.6), 1 mM DTNB, 1 mM EDTA, and 1 mM L-serine. Subsequent to reaction initiation by addition of enzyme at 25°C, the initial velocity was estimated by monitoring the increase in absorbance at 412 nm and the rates were calculated using an extinction coefficient for thionitrobenzoic acid of $\varepsilon = 13600$. A blank

containing all materials except L-Ser was run simultaneously and subtracted from the reaction rate obtained with L-serine. Protein concentrations were determined as described by Khan et al. [9] using BSA (Sigma-Aldrich, United States) as a standard.

Glutathione reductase activity was assayed as described by Anjum et al. [5] by monitoring the GSH-dependent oxidation of NADPH. Its activity was calculated by using extinction coefficient of 6.2/(mM cm). One unit of enzyme was the amount necessary to decompose 1.0 μmol of NADPH/min at 25°C.

Determination of cysteine and glutathione contents. Leaf Cys content was determined spectrophotometrically as described by Khan et al. [9]. Fresh leaves (0.5 g) were homogenized in 5% (w/v) ice-cold perchloric acid (4 ml/g plant tissue). The suspension was centrifuged at 2800 *g* for 1 h at 4°C, and the supernatant was filtered through Whatman no. 30 paper. The filtrate (1 ml) was treated with the acid ninhydrin reagent. The extinction was recorded at 580 nm, and the amount of Cys was calculated using a calibration curve built under similar conditions with a Cys standard.

The contents of reduced (GSH) and oxidized (GSSG) glutathione were estimated as described by Anjum et al. [5]. Fresh leaves (0.5 g) were homogenized in 2 ml of 5% (w/v) sulfosalicylic acid under cold conditions. The homogenate was centrifuged at 10000 *g* for 10 min. To 0.5 ml of the supernatant, 0.6 ml of K-phosphate buffer (100 mM, pH 7.0) and 40 μl of DTNB were added. After 2 min, the absorbance was recorded at 412 nm. GSSG was assayed by the same method in the presence of 2-vinylpyridine, and the GSH concentration was calculated from the difference between total glutathione and GSSG.

Determination of leaf SO_4. Dried leaf powder (100 mg) was digested in a mixture of concentrated HNO_3 and 60% $HClO_4$ (85 : 1, v/v), and the content of SO_4 was estimated using the turbidimetric method of Chesnin and Yien [14].

Statistical analysis. Data were analyzed statistically and given as means ± SE. Analysis of variance (ANOVA) was performed by SPSS (10.0 Inc., United States), and the treatment means were separated using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

RESULTS

Plant Growth and Photosynthetic Traits

Plant dry weight, leaf area, net photosynthesis, and its related variables, such as C_i, F_v/F_m and Chl content were higher in cv. Pusa Bold, the cultivar with a dual type of the S-transporter system at both S sufficient supply and S-deprivation conditions. However, S deprivation significantly affected the growth and photosynthetic traits in both cultivars, to a greater extent in cv. Pusa Jai Kisan. Plant dry weight (PDW), leaf area (LA), P_N, C_i, F_v/F_m and Chl content decreased by 24.1, 24.2, 14.7, 21.5, 12.8 and 31.4%, respectively, in cv. Pusa Bold due to S deprivation as compared to plants supplied with S in sufficient amounts; while in cv. Pusa Jai Kisan, these characteristics decreased by 37.8, 41.4, 22.4, 31.5, 25.4 and 41.7%, respectively, due to S deprivation (Fig. 1).

ATP-Sulfurylase, Serine Acetyltransferase, and Glutathione Reductase Activities

Activities of ATP-S decreased by 14.1% in cv. Pusa Bold due to S deprivation as compared to plants supplied with S in sufficient amount, while in cv. Pusa Jai Kisan, acute depressions were noted in the ATP-S activity (21.8%) due to S deprivation as compared to plants supplied with sufficient S amount (Fig. 2). In contrast, the activity of SAT was significantly higher in cv. Pusa Jai Kisan, the cultivar with LAT. SAT activity enhanced by 4.0 and 2.4% in cvs. Pusa Jai Kisan and Pusa Bold, respectively, due to S deprivation as compared to plants supplied with sufficient S amount. The activity of GR was significantly higher in cv. Pusa Bold, the cultivar with LAT + HAT. GR activity was enhanced by 52.0 and 28.6% in Pusa Bold and Jai Kisan, respectively, due to S deprivation as compared to plants supplied with sufficient S amount (Fig. 2).

Cysteine, Glutathione, and Sulfate Contents

Contents of Cys and GSH decreased by 16.0 and 15.8%, respectively, in cv. Pusa Bold due to S deprivation as compared to plants supplied with S in sufficient amount; while in cv. Pusa Jai Kisan, acute depressions were noted in the contents of Cys (32.3%) and GSH (35.2%) due to S deprivation. In addition, SO_4^{2-} was higher in cv. Pusa Jai Kisan (3.4 mg/kg dry wt) than cv. Pusa Bold (1.89 mg/kg dry wt) under S-deprivation conditions (Fig. 3).

DISCUSSION

Sulfur is an indispensable inorganic plant nutrient. Over the last decades, it has become obvious that S availability is limiting for farming in some parts of the world, including Indian sub-cotenants. Further, sulfate limitation in plants leads to a series of metabolic and physiological responses at adopting plant metabolism to available nutrient supply and to plants machinery for its acquisition to acquire a homeostatic balance [8, 11].

Sulfur limitation has been shown to deplete the pools of reduced S-containing molecules, including Cys, GSH, and/or sulfate; while increased accumulation of *o*-acetylserine (OAS) and serine has been observed in a number of plant species under S limitation [8]. In the present study, cv. Pusa Bold maintained the higher activities of ATP-S along with the higher content of Cys and GSH, while the higher SAT activ-

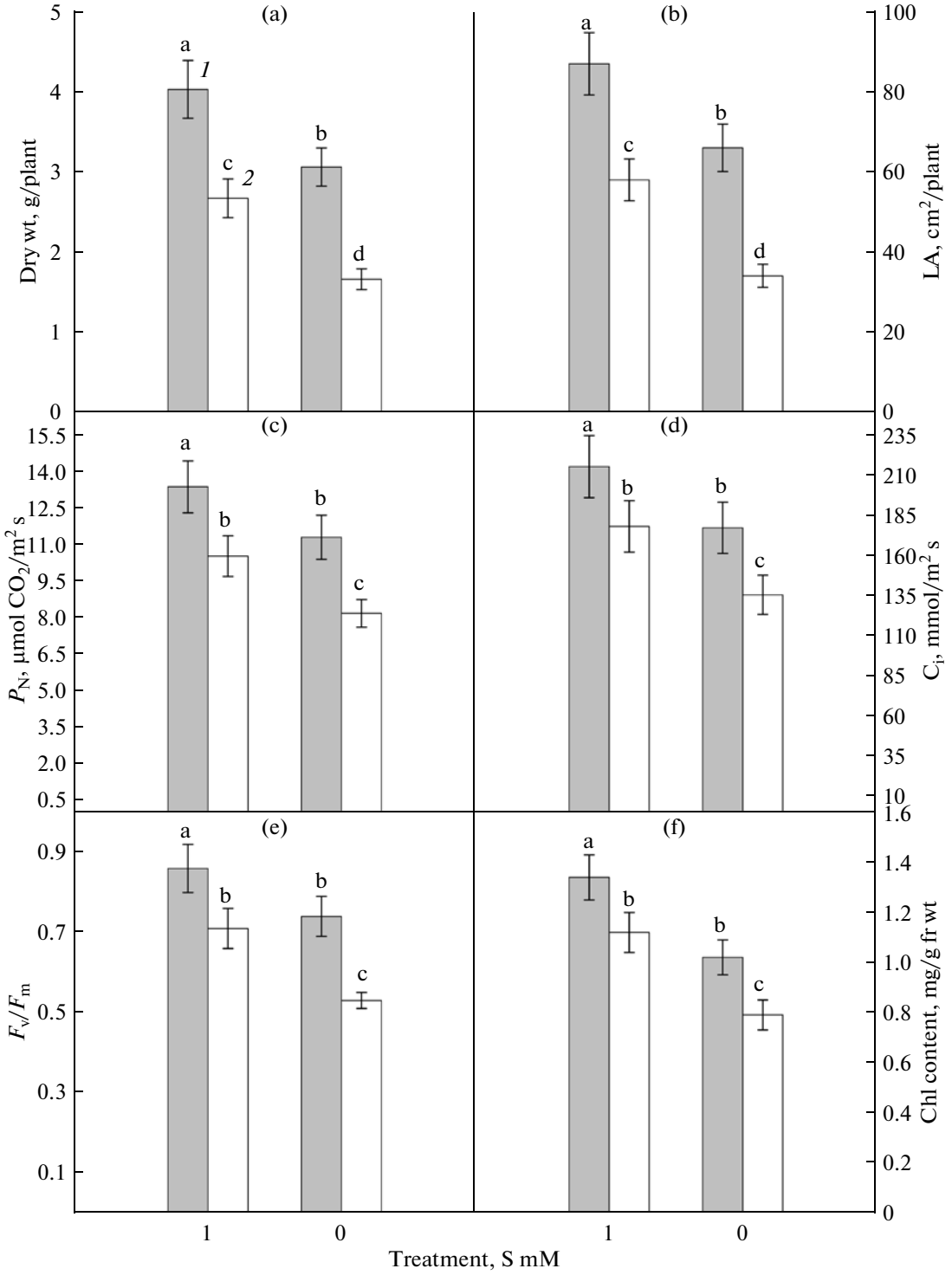

Fig. 1. Effects of S deprivation on plant dry weight, leaf area (LA), net photosynthetic rate (P_N), intracellular CO_2 concentration (C_i), F_v/F_m ratio and Chl content in mustard plants grown at 0 or 1 mM S in 30 days after sowing.

(*1*) Cv. Pusa Bold; (*2*) cv. Pusa Jai Kisan.

Values are means ± SE ($n = 3$) over two independent experiments. Means with similar letters are not significantly different at $P \leq 0.05$, according to Duncan's Multiple Range Test.

ity and the lower content of Cys and GSH was noticed in cv. Pusa Jai Kisan under S-deprivation condition. In fact, ATP-S yields sulfide, which is used for Cys biosynthesis by Cys synthase using OAS, which is synthesized from Ser by SAT, a rate-limiting enzyme for Cys biosynthesis [4, 7]. However, overexpression of SAT in

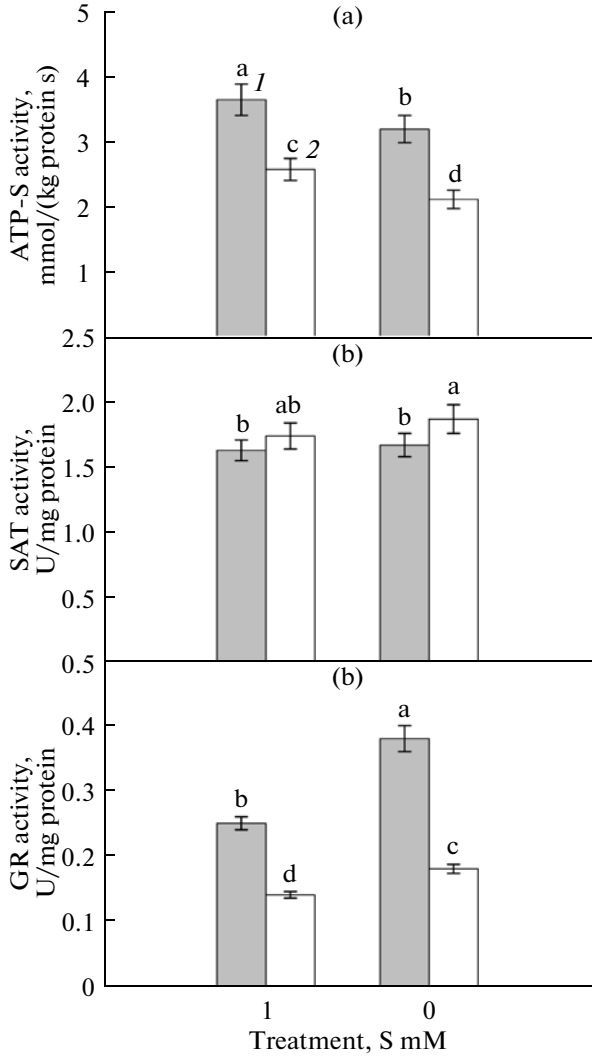

Fig. 2. Effects of S deprivation on activities of ATP sulfurylase (ATP-S), serine acetyltransferase (SAT), and glutathione reductase (GR) in mustard plants grown at 0 or 1 mM S in 30 days after sowing.

(*1*) Cv. Pusa Bold; (*2*) cv. Pusa Jai Kisan.

Values are means ± SE (n = 3) over two independent experiments. Means with similar letters are not significantly different at $P \le 0.05$, according to Duncan's Multiple Range Test.

Nicotiana tabaccum and *Solanum tuberosum* resulted in increased contents of Cys and GSH [15, 16]. Although, an increased SAT activity was noted in cv. Pusa Jai Kisan,this cultivar exhibited the lower activity of ATP-S under S deprivation; therefore, it is obvious, that it will yield fewer sulfides to be incorporated into OAS to result in the Cys and, thus, GSH biosynthesis.

Glutathione is the main redox buffer, which protects the cytosol and other cellular compartments against oxidative stress [4–6]. Since the synthesis of Cys is greatly influenced by the plant S status, it is obvious that it will also affect biosynthesis and thus pool of GSH [2, 16]. A decrease in the reduced GSH

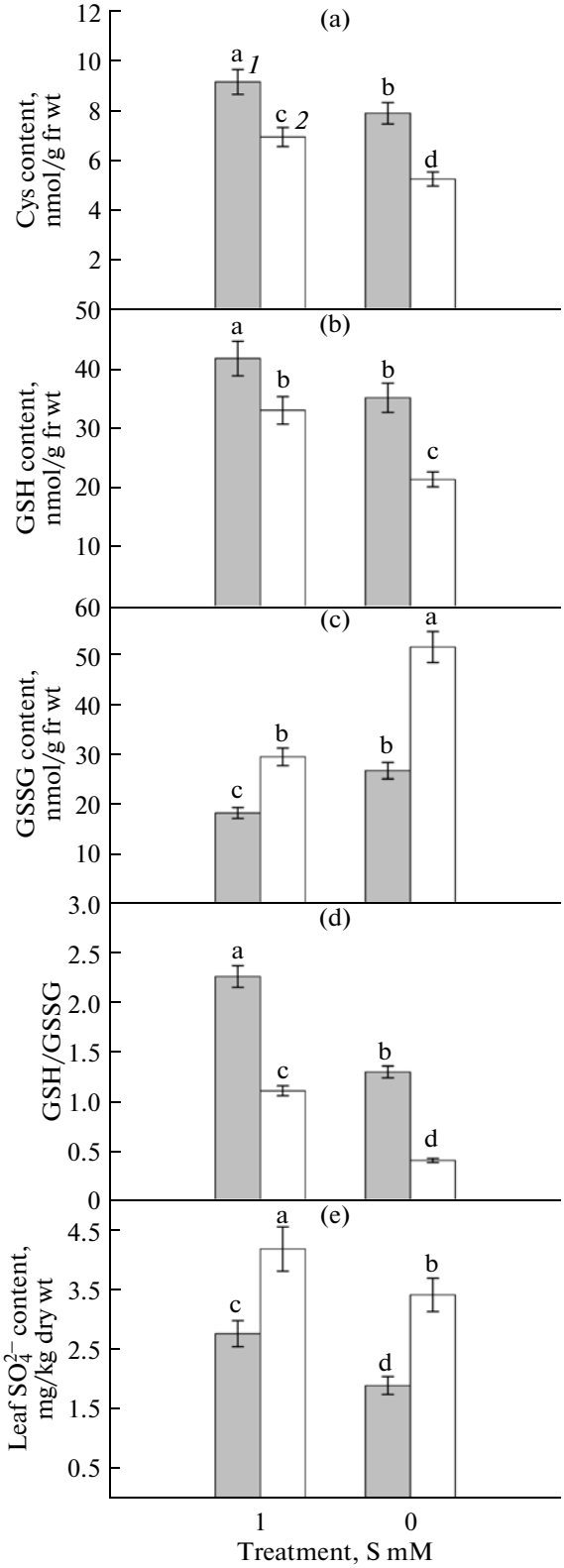

Fig. 3. Effects of S deprivation on the contents of leaf cysteine, glutathione, and sulfate in mustard grown at 0 or 1mM S in 30 days after sowing.

(*1*) Cv. Pusa Bold; (*2*) cv. Pusa Jai Kisan.

Values are means ± SE (n = 3) over two independent experiments. Means with similar letters are not significantly different at $P \le 0.05$, according to Duncan's Multiple Range Test.

and an increase in oxidized GSSG pools under S deprivation occurred to a greater extent in cv. Pusa Jai Kisan, which corroborates well with the findings of Nikiforova et al. [8] obtained for Arabidopsis and Lencioni et al. [17] for rapeseed. In addition, S deprivation increased the oxidised glutathione (GSSG) and up-regulated the activity of GR in both the genotypes, but to a greater extent in cv. Pusa Bold, the cultivar with a dual type of S transporter system. GR catalyzes the NADPH-dependent reduction of GSSG to its reduced form (GSH) and thus maintains the high GSH/GSSG ratio and the activation of CO_2-fixing enzymes [4, 7]. The activity of GR was higher in cv. Pusa Bold, showing its efficiency to maintain the GSH/GSSG homeostasis that led to the protection of the photosynthetic machinery and lesser suppression of photosynthesis under S deprivation.

The cv. Pusa Jai Kisan exhibited the higher content of sulfate in leaves than cv. Pusa Bold, showing that the amount of sulfate that entered the plant system was partially assimilated and/or metabolized. This result is in correspondence with the lower activity ATP-S in cv. Pusa Jai Kisan under S deprivation. In fact, after uptake of sulfate, it can be either stored in the vacuole within the cell or further metabolized in a series of steps in plastids [18]. In contrast, the increased/higher activity of ATP-S in cv. Pusa Bold caused better assimilation of sulfate both under S-deprived and S-sufficient conditions, corroborating well with our earlier finding for moongbean [19].

Sulfur deprivation resulted in suppression of general plant growth, e.g., a decrease in plant dry weight and leaf area, which was more pronounced in cv. Pusa Jai Kisan, the cultivar with LAT, than in cv. Pusa Bold, the cultivar with LAT + HAT transporter system. The S nutrition of a plant influences biomass production and expansion of leaves [20–23]. In addition, the moderate and/or insufficient S availability has been shown to cause acute growth and yield depressions and also impairs the ability of plants to cope with additional stresses [2, 11].

Moreover, S deprivation caused acute depressions in the rate of photosynthesis and its related variables, such as intercellular CO_2, F_v/F_m, and Chl content in both genotypes, to a greater extent in cv. Pusa Jai Kisan. S deprivation caused severe alteration in the primary energy production, e.g., a reduction in F_v/F_m, widely used to detect stress-induced changes in the photosynthetic apparatus. In fact, the F_v/F_m ratio characterizes the maximum quantum efficiency of PSII photochemistry [24]. A decrease in F_v/F_m in both the genotypes can be due to S-deprivation-induced development of slowly relaxing quenching processes and photodamage to PSII reaction centers, both of which thus reduce the maximum quantum efficiency of PSII photochemistry [24]. A similar reduction in F_v/F_m ratio has previously been observed due to S deprivation in rice [10, 24]. Moreover, as also observed in the present study, decreased CO_2 fixation due to S deprivation may be one of reasons for the decrease in PSII photochemistry [15, 24].

In crop plants, photosynthesis is the main driving force for dry matter production [16, 20]. Stronger inhibition of photosynthesis and its related variables in cv. Pusa Jai Kisan due to S deprivation caused a greater reduction in the growth characteristics of this cultivar. A lesser potential of cv. Pusa Jai Kisan in comparison with cv. Pusa Bold was the cumulative result of the effects of S deprivation on photosynthetic traits, oxidative stress protective antioxidants, and ATP-S activity.

In conclusion, the high growth and photosynthesis of Pusa Bold, the cultivar with a dual type of S transporter system, was the result of the coordinate regulation and efficiency of the ATP-S, SAT, and GR enzyme system and a greater Cys and GSH production. Under S-deprivation cv. Pusa Bold showed increased activities of ATP-S and GR, a greater capacity of Cys and GSH production, and maintenance of the GSH/GSSG and F_v/F_m ratios. These characteristics of cv. Pusa Bold helped in protecting the photosynthetic apparatus, thus maintaining active growth and the high dry weight yield. Despite the high SAT activity in cv. Pusa Jai Kisan, the cultivar with the low-affinity S transporter system, it could not translated into increased Cys and GSH production, thus resulting in a suppressed growth and photosynthesis under S deprivation.

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